

**INHIBITION OF NF- $\kappa$ B ACTIVATION BY BLOCKADE OF IKK $\beta$ -NEMO  
INTERACTIONS AT THE NEMO BINDING DOMAIN**

**INVENTORS**

5 Michael J. May and Sankar Ghosh

**RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Patent Application Serial No.  
60/201,261 filed May 2, 2000 which is herein incorporated by reference in its entirety.

**U.S. GOVERNMENT SUPPORT**

This work was supported by a grant from the National Institute of Health  
(AI33443).

**FIELD OF THE INVENTION**

The invention relates to compositions and methods for the selective inhibition of  
cytokine-mediated NF- $\kappa$ B activation by blocking the interaction of NEMO with I $\kappa$ B  
kinase- $\beta$  (IKK $\beta$ ) at the NEMO binding domain (NBD). The blockade of IKK $\beta$ -NEMO  
interaction results in inhibition of IKK $\beta$  kinase activation and subsequent decreased  
phosphorylation of I $\kappa$ B. Phosphorylation of I $\kappa$ B is an integral step in cytokine-mediated  
NF- $\kappa$ B activation.

**BACKGROUND OF THE INVENTION**

NF- $\kappa$ B is a transcription factor which mediates extracellular signals responsible  
for induction of genes involved in pro-inflammatory responses (see Baltimore *et al.*,  
(1998) U.S. Patent No. 5,804,374). NF- $\kappa$ B is anchored in the cytoplasm of most  
non-stimulated cells by a non-covalent interaction with one of several inhibitory proteins  
known as I $\kappa$ Bs (see May & Ghosh, (1997) Semin. Cancer. Biol. 8, 63-73; May & Ghosh,

(1998) Immunol. Today 19, 80-88; Ghosh *et al.*, (1998) Annu. Rev. Immunol. 16, 225-260). Cellular stimuli associated with pro-inflammatory responses such as TNF $\alpha$ , activate kinases, which in turn activate NF- $\kappa$ B by phosphorylating I $\kappa$ Bs. The kinases that phosphorylate I $\kappa$ Bs are called I $\kappa$ B kinases (IKKs).

5           Phosphorylation targets I $\kappa$ Bs for ubiquitination and degradation. The degradation and subsequent dissociation of I $\kappa$ Bs from NF- $\kappa$ B reveals the nuclear localization signal on NF- $\kappa$ B, resulting in nuclear translocation of active NF- $\kappa$ B, leading to up-regulation of genes responsive to NF- $\kappa$ B (May & Ghosh, (1997) Semin. Cancer. Biol. 8, 63-73; May & Ghosh, (1998) Immunol. Today 19, 80-88; Ghosh *et al.*, (1998) Annu. Rev. Immunol. 16,  
10       225-260; Siebenlist *et al.*, (1994) Annu. Rev. Cell Biol. 12, 405-455). Phosphorylation of I $\kappa$ Bs is therefore an essential step in the regulation of NF- $\kappa$ B mediated pro-inflammatory responses.

          The identification and characterization of kinases that phosphorylate I $\kappa$ Bs have led to a better understanding of signaling pathways involving NF- $\kappa$ B activation. Several  
15       different subtypes of IKK have been identified thus far. IKK $\alpha$  was initially identified as an I $\kappa$ B kinase induced by TNF $\alpha$  stimulation in HeLa cells (DiDonato *et al.*, (1997) Nature 388, 548-554). Another I $\kappa$ B kinase homologous to IKK $\alpha$  was identified, termed IKK $\beta$  and determined to be the major I $\kappa$ B kinase induced following TNF $\alpha$  stimulation  
20       (Takeda *et al.*, (1999) Science 284, 313-316; Hu *et al.*, (1999) Science 284, 316-320; Li *et al.*, (1999) Science 284, 321-325; Pot *et al.*, (2000) U.S. Patent No. 6,030,834; Woronicz & Goeddel (1999) U.S. Patent No. 5,939,302). IKK $\alpha$  and IKK $\beta$  have 52% overall homology and 65% homology in the kinase domain (Zandi *et al.*, (1997) Cell 91, 243-252).

          I $\kappa$ B protein kinases (IKKs) phosphorylate I $\kappa$ Bs at specific serine residues. For  
25       example, they specifically phosphorylate serines 32 and 36 of I $\kappa$ B $\alpha$  (Traenckner *et al.*, (1995) EMBO J. 14, 2876-2883; DiDonato *et al.*, (1996) Mol. Cell. Biol. 16, 1295-1304). Phosphorylation of both sites is required to efficiently target I $\kappa$ B $\alpha$  for degradation. Furthermore, activation of IKK $\alpha$  and IKK $\beta$  is usually in response to NF- $\kappa$ B activating

agents and mutant IKK $\alpha$  and IKK $\beta$ , which are catalytically inactive, can be used to block NF- $\kappa$ B stimulation by cytokines such as TNF $\alpha$  and IL-1 (Régnier *et al.*, (1997) Cell 90, 373-383; Delhase *et al.*, (1999) Science 284, 309-313). I $\kappa$ B protein kinases are therefore essential in regulation of NF- $\kappa$ B activation processes.

5           IKK $\alpha$  and IKK $\beta$  have distinct structural motifs including an amino terminal serine-threonine kinase domain separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper domain. These structural characteristics are unlike other kinases, and the non-catalytic domains are thought to be involved in protein-protein interactions. Proteins which bind to IKKs may therefore be capable of regulating the  
10           activity of NF- $\kappa$ B (Marcu *et al.*, (1999) U.S. Patent No. 5,972,655) and potentially regulating downstream events such as induction of NF- $\kappa$ B. For instance, NEMO (NF- $\kappa$ B Essential Modulator) is a protein which has been identified to bind to IKKs and facilitate kinase activity (Yamaoka *et al.*, (1998) Cell 93, 1231-1240; Rothwarf *et al.*, (1998) Nature 395, 287-300; Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Haraj &  
15           Sun, (1999) J. Biol. Chem. 274, 22911-22914; Jin & Jeang, (1999) J. Biomed. Sci. 6, 115-120).

          The discovery of agents capable of modulating IKK binding proteins involved in the regulation of NF- $\kappa$ B induction will be useful for controlling NF- $\kappa$ B mediated pro-inflammatory processes. A particular advantage of such agents is that while blocking NF-  
20            $\kappa$ B induction via IKK, they would not inhibit the basal activity of NF- $\kappa$ B. This invention provides for the identification and characterization of agents which modulate IKK binding proteins such as the NF- $\kappa$ B Essential Modulator (NEMO) protein.

## SUMMARY OF THE INVENTION

25           This invention provides compositions and methods for the selective inhibition of cytokine-mediated NF- $\kappa$ B activation by blocking the interaction of NEMO with I $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) at the NEMO binding domain (NBD).

In one aspect, the present invention provides methods of inhibiting NF- $\kappa$ B induction in a cell which includes administering an effective amount of a peptide which blocks the interaction of one or more IKKs and NEMO. In one specific embodiment, this invention includes such methods wherein the IKKs are IKK $\alpha$  and/or IKK $\beta$ . In another  
5 specific embodiment of this invention, the peptides include at least one NEMO binding domain. In still another specific embodiment of this invention, such methods utilize peptides which include at least one membrane translocation domain. In still another specific embodiment of this invention, the peptides utilized in such methods include amino acid sequences comprising the sequences of SEQ ID NO: 2, 4, 5, 6, 11, 12, 16, 17  
10 or 18.

In another aspect, the present invention provides methods of inhibiting inflammation in a mammal by administering an effective amount of a peptide which blocks the interaction of IKK and NEMO. In one specific embodiment, such methods include peptides which block the recruitment of leukocytes into sites of acute and chronic  
15 inflammation. In another specific embodiment, the methods of the present invention include peptides which down-regulate the expression of E-selectin on leukocytes. In yet another embodiment, the methods include peptides which block osteoclast differentiation.

In another aspect, the present invention provides methods of inhibiting NF- $\kappa$ B-dependent target gene expression in cells by administering effective amounts of a peptide  
20 which block the interaction of one or more IKKs and NEMO. In one specific embodiment, this invention provides such methods wherein the IKK is IKK $\beta$ . In another specific embodiment, this invention provides such methods wherein the NF- $\kappa$ B-dependent target gene is E-selectin.

In another aspect, the present invention provides methods of identifying agents  
25 which interact with the NEMO binding domain wherein the methods include the steps of:  
(a) exposing cells which express NEMO and NF- $\kappa$ B to the agents; and

002280"092E4950

(b) determining whether the agents modulate activation of NF- $\kappa$ B by the cell, wherein an alteration in activation of NF- $\kappa$ B is indicative of agents which interact with the NEMO binding domain.

In another aspect, the present invention provides methods of identifying agents which modulate the activity of NEMO which includes the steps of:

(a) exposing cells which express NEMO to the agents; and

(b) determining whether the agents modulate the activity of NEMO, thereby identifying agents which modulate the activity of NEMO.

In another aspect, the present invention provides fusion peptides which include the NEMO binding domain and at least one membrane translocation domain. In a preferred embodiment, the fusion peptide membrane translocation domain facilitates membrane translocation *in vivo*. In a specific embodiment, this invention provides such fusion peptides wherein the membrane translocation domain is either the third helix of the *antennapedia* homeodomain or the HIV-1 Tat protein. In another specific embodiment, this invention provides such fusion peptides wherein the NEMO binding domain is SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19. In still another aspect, this invention provides compositions which include such fusion peptides. In yet another embodiment, this invention provides such compositions which also include one or more carriers.

In another aspect, the present invention provides isolated peptides such as the following:

(a) an isolated peptide which includes the amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19

(b) an isolated peptide which includes a fragment of at least three amino acids of an amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19;

(c) an isolated peptide which includes conservative amino acid substitutions of the amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19; and

(d) naturally occurring amino acid sequence variants of amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19. In still another aspect, this invention provides compositions which include such isolated peptides. In yet another embodiment, this invention provides such compositions which also include one or more carriers.

In another aspect, this invention provides isolated peptides which consist of the amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19.

## DESCRIPTION OF THE DRAWINGS

**Figure 1 - NEMO interacts with the COOH-terminus of IKK $\beta$ .** (A) GST alone or GST-NEMO were precipitated from bacterial lysates using glutathione-agarose, separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of GST or GST-NEMO were used in subsequent GST pull-down experiments. The scheme depicted in the right panel represents the COOH- and NH<sub>2</sub>-terminal truncation mutants of IKK $\beta$  used to determine the region of NEMO interaction. (B) IKK $\beta$  mutants were cloned, expressed by *in vitro* translation (input; left panel) and used for GST pull-down (right panel). (C) Wild-type IKK $\beta$  and IKK $\beta$ -(644-756) were *in vitro* translated (left panel) and used for GST pull-down analysis (left panel). (D) HeLa cells were transiently transfected with either vector alone or increasing concentrations (0.25, 0.5, 1.0  $\mu$ g/ml) of the xpress-tagged IKK $\beta$ -(644-756) construct together with the pBIIX-luciferase reporter plasmid. After forty-eight hours cells were treated with either TNF $\alpha$  (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for four hours then NF- $\kappa$ B activity was measured. Western blot analysis from portions of the lysate using anti-xpress (inset) demonstrates the increasing levels of expressed protein.

**Figure 2 - The first  $\alpha$ -helical region of NEMO is required for binding to IKK $\beta$ .**

(A) A truncated version of IKK $\beta$  consisting of only the COOH-terminus from residue V644 to S756 was fused with GST (GST-644-756) and expressed in bacteria. After precipitation by glutathione agarose, GST alone and GST-(644-756) were separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of each protein were used for subsequent GST pull-down analyses. Various NH<sub>2</sub>- and COOH-terminal truncations of NEMO were constructed, [<sup>35</sup>S]-methionine labeled and used for *in vitro* pull down (right panel). Mutants that interacted with GST-(644-756) are indicated (+). None of the mutants interacted with GST alone. (B) Wild-type NEMO and a deletion mutant lacking the first  $\alpha$ -helical region (del. $\alpha$ H) were *in vitro* translated (left panel: input) and used for GST pull-down using the proteins shown above (A: left). (C) HeLa cells were transfected with pBIIx-luciferase together with either pcDNA-3 (vector) or increasing concentrations of del. $\alpha$ H (0.25, 0.5, 1.0  $\mu$ g/ml) for forty-eight hours then treated for four hours with TNF $\alpha$  (10 ng/ml). Cells were then lysed and NF- $\kappa$ B activity was measured by luciferase assay.

**Figure 3 - Interaction with NEMO and functional kinase activity requires an**

**IKK $\alpha$ -homologous region of the IKK $\beta$  COOH-terminus.** (A) Truncation mutations of IKK $\beta$  sequentially omitting the extreme COOH-terminus (1-733), the serine-free region (1-707), the serine rich-domain (1-662) and the  $\alpha$ <sub>1</sub>-region (1-644) were expressed and labeled by *in vitro* translation and used for GST pull-down by GST-NEMO (Figure 1A). None of the mutants interacted with GST alone. (B) Sequence alignment of the extreme COOH-termini of IKK $\beta$  and IKK $\alpha$ . The  $\alpha$ <sub>2</sub>- and glutamate-rich regions are indicated and the six identical amino acids are shaded. (C) Wild-type IKK $\beta$  and the truncation mutants (1-733 and 1-744) were [<sup>35</sup>S]-methionine-labeled (input) and used for *in vitro* pull down with either GST alone or GST-NEMO. (D) HeLa cells were transfected for forty-eight hours with 1  $\mu$ g/well of the indicated FLAG-tagged constructs followed by immunoprecipitation using anti-FLAG. The immunoprecipitates were incubated in

kinase buffer containing [<sup>32</sup>P]-labeled γATP for fifteen minutes at 30°C then washed with lysis buffer containing 1% Triton-100. Resulting complexes were separated by SDS-PAGE (10%) and visualized by autoradiography (upper panel). The lower panel is an immunoblot from identical samples demonstrating equivalent amounts of transfected protein in each lane. (E) HeLa cells were transfected for 48 hours with 1 μg/ml of the indicated constructs or empty vector (pcDNA-3) together with pBIIx-luciferase. NF-κB activity was determined by luciferase assay. (F) HeLa cells transfected for forty-eight hours with FLAG-tagged versions of either IKKβ (wild-type) or IKKβ-(1-733) were either untreated (-) or treated for seven minutes (+) with TNFα (10 ng/ml). Following lysis and immunoprecipitation using anti-FLAG, immune-complex kinase assay (upper panels) was performed. Identical samples were immunoprecipitated and immunoblotted with anti-FLAG (lower panels).

Figure 4 - Association of NEMO with IKKβ and IKKα reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected with vector alone, FLAG-tagged IKKα or IKKβ (1 μg/well) or xpress-tagged NEMO (1 μg/well) to a total DNA concentration of 2 μg/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) Wild-type IKKα and IKKα-(1-737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (C) Full length cDNA encoding human IKK<sub>α</sub> was obtained by RT-PCR from HeLa cell mRNA using the Expand™ Long Template PCR System (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and cloned into the *EcoRI* and *XbaI* sites of pcDNA-3. GST pull-down analysis was



performed using [<sup>35</sup>S]-methionine-labeled IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ . (D) A deletion mutant of IKK $\beta$  lacking the NBD (del.NBD) was [<sup>35</sup>S]-methionine-labeled (input) and used for GST pull down analysis. (E) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (N721-S756) of human IKK $\beta$  was generated using MacVector™ (version 6.5.3) software. The NBD (L737-L742) is boxed. (F) COS cells were transfected for forty-eight hours with a total of 2  $\mu$ g DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKK $\beta$ -(1-744) containing point mutations within the NBD as indicated. Following lysis and immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (G) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NF $\kappa$ B activity in lysate was measured by luciferase assay.

Figure 5 - A cell-permeable peptide spanning the IKK $\beta$  NBD inhibits the IKK $\beta$ /NEMO interaction, TNF $\alpha$ -induced NF- $\kappa$ B activation and NF- $\kappa$ B-dependent gene expression. (A) GST-pull-down analysis was performed using either GST-NEMO-*in vitro* translated IKK $\beta$  (upper panel) or GST-IKK $\beta$ -(644-756)-*in vitro* translated NEMO (lower panel). The assay was performed in the absence (no peptide) or presence of increasing concentrations (125, 250, 500 or 1000  $\mu$ M) of either mutant (MUT) or wild-type (WT) NBD peptide. (B) HeLa cells were incubated with either peptide (200  $\mu$ M) for the times indicated. Following lysis, the IKK complex was immunoprecipitated using anti-NEMO and the resulting immunoblot probed with anti-IKK $\beta$ . (C) HeLa cells were transfected for forty-eight hours with pBIIX-luciferase then incubated for two hours in the absence (control) or presence of mutant or wild-type NBD peptide (100 and 200  $\mu$ M of each). Subsequently the cells were either treated with TNF $\alpha$  (10 ng/ml) as indicated (left panel) or left untreated (right panel) for a further four hours after which NF- $\kappa$ B activation was measured by luciferase assay. (D) HeLa cells were incubated for three hours with

increasing concentrations (50, 100 or 200  $\mu$ M) of each peptide followed by treatment for fifteen minutes with TNF $\alpha$  (10 ng/ml) as indicated (+). Following lysis, nuclear extracts were made and 10  $\mu$ g of protein from each sample was used for EMSA using a specific [<sup>32</sup>P]-labeled  $\kappa$ B-site probe. (E) Primary HUVEC were pre-incubated for two hours with of wild-type (left) or mutant (right) NBD peptides (100  $\mu$ M) then stimulated with TNF $\alpha$  (10 ng/ml) for a further six hours. Control cells received no peptide. Cells were stained with either anti-E-selectin (H4/18) or a non-binding control antibody (K16/16) and expression was measured by FACS (FACSort, Becton Dickinson). The profiles show E-selectin staining in the absence (shaded) and presence (solid line) of TNF $\alpha$  and control antibody staining under the same conditions (dashed line, no TNF $\alpha$ ; dotted line, TNF $\alpha$ ).

Figure 6 - The wild-type NBD peptide inhibits NF- $\kappa$ B-induced gene expression and experimentally induced inflammation. (A) PMA-induced ear edema in mice topically treated with either vehicle (VEH), dexamethasone (DEX) or NBD peptides was induced and measured as described in Example 8. Data are presented as mean differences in ear thickness  $\pm$  SD (\* =  $p < 0.05$  compared with both untreated control [-] and vehicle [VEH]). (B) The effects of the NBD peptide compared with the effect of dexamethasone (DEX) on Zymosan (ZYM)-induced peritonitis in mice were determined as described again in Example 8. Control mice were injected with phosphate-buffered saline (PBS).

Figure 7 - Dose dependent inhibition of osteoclast differentiation by wild-type but not mutant NBD peptides. Data are presented as the mean determination of triplicate samples  $\pm$  SD.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

### I. General Description

The invention relates generally to compositions and methods for regulation of cytokine-induced NF- $\kappa$ B activation associated with pro-inflammatory responses.

Specifically, the invention relates to compositions and methods for blocking the interaction of NEMO with IKK $\beta$  at the NEMO binding domain (NBD), thereby inhibiting phosphorylation, degradation and subsequent dissociation of I $\kappa$ B from NF- $\kappa$ B. This inhibition results in blockade of NF- $\kappa$ B activation associated with pro-inflammatory responses.

The invention also includes methods for screening and identifying agents capable of interacting with the NBD thereby blocking the interaction of NEMO with IKK $\beta$ . The invention further includes the use of these agents in the treatment of disorders associated with regulation of NF- $\kappa$ B induction.

## II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, the term "binding" refers to the adherence of molecules to one another, such as, but not limited to, enzymes to substrates, antibodies to antigens, DNA strands to their complementary strands. Binding occurs because the shape and chemical nature of parts of the molecule surfaces are "complementary". A common metaphor is the "lock-and-key" used to describe how enzymes fit around their substrate.

The term "fusion peptide" or "fusion polypeptide" or "fusion protein" refers to a peptide, polypeptide or protein that is obtained by combining two distinct amino acid sequences. Typically, a partial sequence from one peptide, polypeptide or protein is linked to another heterologous peptide, polypeptide or protein, using cDNA technology.

The terms "conservative variation" or "conservative substitution" as used herein refers to the replacement of an amino acid residue by another, biologically similar residue. Conservative variations or substitutions are not likely to change the shape of the

peptide chain. Examples of conservative variations, or substitutions, include the replacement of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like.

5 As used herein, the term “domain” refers to a part of a molecule or structure that shares common physicochemical features, such as, but not limited to, hydrophobic, polar, globular and helical domains or properties. Specific examples of binding domains include, but are not limited to, DNA binding domains and ATP binding domains.

10 As used herein, the term “membrane translocation domain” refers to a peptide capable of permeating the membrane of a cell and which is used to transport attached peptides into a cell *in vivo*. Membrane translocation domains include, but are not limited to, the third helix of the *antennapedia* homeodomain protein (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450; U.S. Patent No. 5,888,762; U.S. Patent No. 6,015,787) and the HIV-1 protein Tat (Lindgren *et al.*, (2000) Trends Pharmacol. Sci. 21, 99-103).

15 As used herein, the term “I $\kappa$ B” (I kappa B) refers to any one of several members of a family of structurally related inhibitory proteins that function in the regulation of NF- $\kappa$ B induction.

20 As used herein, the term “I $\kappa$ B-kinase” or “I $\kappa$ B protein kinase” or “I $\kappa$ B-kinase complex” or “I $\kappa$ B protein kinase complex” or “IKK” refers to a kinase that phosphorylates I $\kappa$ Bs.

As used herein, the term “IKK $\alpha$ ” refers to the  $\alpha$  subunit of an I $\kappa$ B-kinase complex.

As used herein, the term “IKK $\beta$ ” refers to the  $\beta$  subunit of an I $\kappa$ B-kinase complex.

As used herein, the term “NEMO” (NF- $\kappa$ B Essential Modulator), “IKK $\gamma$ ” or “IKKAP” refers to the protein which binds to IKKs and facilitates kinase activity.

25 As used herein, the term “NEMO Binding Domain” or “NBD” refers to the  $\alpha$ <sub>2</sub>-region (residues 737-742) of wild-type IKK $\beta$ , or the corresponding six amino acid sequence of wild-type IKK $\alpha$  (residues 738-743) which are critical for interaction with NEMO. The nucleic acid sequence and the corresponding amino acid sequence of the

wild-type NBD are provided in SEQ ID NO: 1 (GenBank Accession No. AR067807; nucleotides 2203-2235) and SEQ ID NO: 2, respectively.

As used herein, a "peptide mimetic" of a known polypeptide refers to a compound that mimics the activity of the peptide or polypeptide, but which is composed of molecules other than, or in addition to, amino acids.

As used herein, the term "wild-type" refers to the genotype and phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the genotype and phenotype of a mutant.

### III. Specific Embodiments

#### A. Agents which block NEMO interaction with IKK $\beta$

The identification of the NBD on IKK $\beta$  allows for the discovery of compounds that are capable of down-regulating the activity of IKK $\beta$ , for example, modulating its interaction with the protein NEMO. Molecules that down-regulate interaction of NEMO with the IKK complex are therefore part of the invention. Down-regulation is defined here as a decrease in activation, function or synthesis of NEMO, its ligands or activators. It is further defined to include an increase in the degradation of the NEMO gene, its protein product, ligands or activators. Down-regulation is therefore achieved in a number of ways. For example, administration of molecules that can destabilize the binding of NEMO to IKK $\beta$ . Such molecules encompass peptide products, including those encoded by the DNA sequences of the NEMO gene or DNA sequences containing various mutations. These mutations may be point mutations, insertions, deletions or spliced variants of the IKK $\beta$  gene encoding the NBD. This invention also includes truncated peptides encoded by the DNA molecules described above. These peptides being capable of interfering with interaction of NEMO and IKK $\beta$ .

Molecules that block the phosphorylation of I $\kappa$ B and subsequent degradation of this protein can also be used to down-regulate NF- $\kappa$ B functions and are within the scope of the invention. Phosphorylation of I $\kappa$ B by IKK $\beta$  results in ubiquitination and

002280-09221950

degradation of I $\kappa$ B and subsequent dissociation of I $\kappa$ B, allowing for nuclear translocation of NF- $\kappa$ B, leading to up-regulation of genes critical to the inflammatory response. IKK $\beta$  inhibitors which interact at the NBD may therefore be used to down-regulate NF- $\kappa$ B function. Down-regulation of NF- $\kappa$ B may also be accomplished by the use of polyclonal or monoclonal antibodies or fragments thereof directed against the NBD on IKK $\beta$ . Such molecules are within the claimed invention. This invention further includes small molecules with the three-dimensional structure necessary to bind with sufficient affinity at the NBD to block NEMO interactions with IKK $\beta$ . IKK $\beta$  blockade resulting in decreased degradation of I $\kappa$ B and decreased activation of NF- $\kappa$ B make these small molecules useful as therapeutic agents in blocking inflammation.

The invention further provides binding agents specific to NEMO, capable of blocking interaction of NEMO at the NBD on IKK $\beta$ . Such agents include substrates, agonists, antagonists, natural intracellular binding targets, etc. The invention also provides methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, *e.g.* NF- $\kappa$ B activation. Novel NEMO-specific binding agents include peptides comprising the NBD of IKK $\beta$  (residues 737-742), NEMO-specific receptors, such as somatically recombined peptide receptors like specific antibodies or T-cell antigen receptors (see Harlow & Lane, (1988) *Antibodies - A Laboratory Manual*, Cold Spring Harbor Laboratory Press) and other natural intracellular binding agents identified with assays such as one, two and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate IKK $\beta$  function, *e.g.* IKK $\beta$ -dependent transcriptional activation of NF- $\kappa$ B. For example, inhibitors of IKK $\beta$  activity may be used to regulate signal transduction involving I $\kappa$ B. Exemplary IKK $\beta$  inhibitors include competitive inhibitors of NEMO binding at the NBD, for example, the peptide set forth in SEQ ID

NO: 2 and conservative substitutions thereof. Conservative substitutions of amino acid residues of the peptide set forth in SEQ ID NO: 2 at positions 737, 740 and 742 are also encompassed in the invention (see Table 1 for examples of conservative substitutions which have no significant effect on NEMO binding at NBD).

Accordingly, the invention provides methods for modulating signal transduction involving I $\kappa$ B in a cell comprising the step of modulating IKK $\beta$  activity, *e.g.* by contacting the cell with an agent which inhibits the interaction of NEMO with IKK $\beta$  at the NBD, thereby inhibiting IKK $\beta$  function. The cell may reside in culture or *in situ*, *i.e.* within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other IKK $\beta$  binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

The agents discussed above represent various effective therapeutic compounds in blocking NEMO-IKK $\beta$  interactions and thus regulating NF- $\kappa$ B-mediated processes. Applicants have thus provided antagonists and methods of identifying antagonists that are capable of down-regulating IKK $\beta$ .

#### B. Screening Assays

In addition, this invention also provides compounds and methods of screening for compounds that block the function, prevent the synthesis or reduce the biologic stability of IKK $\beta$  by interacting at the NBD. Biologic stability is a measure of the time between the synthesis of the molecule and its degradation. For example, the stability of a protein, peptide or peptide mimetic (Kauvar, Nature Biotech. (1996) 14, 709) therapeutic may be shortened by altering its sequence to make it more susceptible to enzymatic degradation.

The present invention also includes methods of screening for agents which deactivate, or act as antagonists of IKK $\beta$  function. Such agents may be useful in the

modulation of pathological conditions associated with alterations in IKK $\beta$  or NF- $\kappa$ B protein levels.

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of proteins of the invention. For example, agents which interact with IKK $\beta$  at the NBD, or interact with NEMO, thereby blocking NEMO interaction with IKK $\beta$ . In detail, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance the entire IKK $\beta$  peptide can be used. Alternatively, a fragment of the protein can be used. For example, the peptide fragment comprising NBD can be used to block interaction of IKK $\beta$  with NEMO.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with either IKK $\beta$  or NEMO under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For



example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density-sediment centrifugation can be used.

5           After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

10           To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide-binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins. Alternatively, binding partners may be  
15           identified using a Far-Western assay according to the procedures of Takayama *et al.*, (1997) *Methods Mol. Biol.* 69, 171-184 or Sauder *et al.*, (1996) *J. Gen. Virol.* 77, 991-996 or identified through the use of epitope tagged proteins or GST fusion proteins.

20           Alternatively, the nucleic acid molecules encoding the peptides of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described (see for example, Stratagene Hybrizap® two-hybrid system).

          Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of NEMO or IKK $\beta$ . Such methods or assays may utilize any means of monitoring or detecting the desired activity.

25           In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell

populations. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysate may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysate are then analyzed with the probe.

5           Antibody probes are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides. Peptides or proteins comprising the NBD are of sufficient length, or if desired, as required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some  
10           circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co. may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of  
15           the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

          While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is  
20           preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler & Milstein, (1992) Biotechnology 24, 524-526 or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten, peptide or protein.  
25           When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

          The desired monoclonal antibodies may be recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera

which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab' of F(ab')<sub>2</sub> fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

5           The antibodies or fragments may also be produced, using current technology, by recombinant means. Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin.

          Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected  
10       when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

          As used herein, an agent is said to be rationally selected or designed when the  
15       agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that comprises the NBD on IKK $\beta$  or the IKK $\beta$  binding domain on NEMO. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the  
20       amino acid sequence of SEQ ID NO: 2 or a peptide with conservative substitutions thereof.

          The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present  
25       invention.

          The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide

synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

The present invention further provides isolated nucleic acid molecules that encode the peptide having the NBD and conservative nucleotide substitutions thereof, preferably in isolated form. Conservative nucleotide substitutions include nucleotide substitutions which do not effect the coding for a particular amino acid as most amino acids have more than one codon (see King & Stansfield (Editors), A Dictionary of Genetics, Oxford University Press, 1997 at page 19). Conservative nucleotide substitutions therefore also include silent mutations and differential codon usage. For example, the invention includes the nucleic acid (SEQ ID NO: 1) encoding the peptide set forth in SEQ ID NO: 2, and conservative nucleotide substitutions thereof. The invention also includes nucleic acids encoding the peptides set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 and conservative nucleotide substitutions thereof. Any nucleic acid that encodes the peptides set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 is encompassed in the invention, given the multiple permutations of nucleotide sequences possible which encode these peptides.

Specific examples of nucleic acids encompassed by this invention include, but are not limited to the following: (1) the amino acids of the peptide of SEQ ID NO: 2 can be encoded by the nucleic acid sequence TTAGATTGGTCTTGGTTA (SEQ ID NO: 24) or TTGGACTGGTCCTGGCTA (SEQ ID NO: 25); and (2) the amino acids of the peptide of SEQ ID NO: 15 can be encoded by the nucleic acid sequence TTAGATTGGTCTTATCTG (SEQ ID NO: 26) or CTTGACTGGTCATACTTA (SEQ ID NO: 27).

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. Modifications to the primary structure of the nucleic acid itself by deletion, addition, or alteration of the amino acids incorporated

into the protein sequence during translation can be made without destroying the activity of the peptide. Such substitutions or other alterations result in peptide having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

5 Another class of agents of the present invention are antibodies immunoreactive with critical positions of proteins of the invention. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

#### 10 C. High Throughput Assays

Introduction - The power of high throughput screening is utilized to the search for new compounds which are capable of interacting with the NEMO binding domain. For general information on high-throughput screening, see, for example, Cost-Effective Strategies for Automated and Accelerated High-Throughput Screening, IBCS Biomedical Library Series, IBC United States Conferences, 1996; Devlin (Editor), High Throughput Screening, Marcel Dekker 1998; U.S. Patent No. 5,763,263. High throughput assays utilize one or more different assay techniques.

1. Immunodiagnosics and Immunoassays - These are a group of techniques used for the measurement of specific biochemical substances, commonly at low concentrations in complex mixtures such as biological fluids, that depend upon the specificity and high affinity shown by suitably prepared and selected antibodies for their complementary antigens. A substance to be measured must, of necessity, be antigenic - either an immunogenic macromolecule or a haptenic small molecule. To each sample a known, limited amount of specific antibody is added and the fraction of the antigen combining with it, often expressed as the bound:free ratio, is estimated, using as indicator a form of the antigen labeled with radioisotope (radioimmunoassay), fluorescent molecule (fluoroimmunoassay), stable free radical (spin immunoassay), enzyme (enzyme immunoassay), or other readily distinguishable label.

002280"092E4960

Antibodies can be labeled in various ways, including: enzyme-linked immunosorbent assay (ELISA); radioimmuno assay (RIA); fluorescent immunoassay (FIA); chemiluminescent immunoassay (CLIA); and labeling the antibody with colloidal gold particles (immunogold).

5 Common assay formats include the sandwich assay, competitive or competition assay, latex agglutination assay, homogeneous assay, microtitre plate format and the microparticle-based assay.

10 Enzyme-linked immunosorbent assay (ELISA) - ELISA is an immunochemical technique that avoids the hazards of radiochemicals and the expense of fluorescence detection systems. Instead, the assay uses enzymes as indicators. ELISA is a form of quantitative immunoassay based on the use of antibodies (or antigens) that are linked to an insoluble carrier surface, which is then used to "capture" the relevant antigen (or antibody) in the test solution. The antigen-antibody complex is then detected by measuring the activity of an appropriate enzyme that had previously been covalently  
15 attached to the antigen (or antibody).

For information on ELISA techniques, see, for example, Crowther, ELISA: Theory and Practice (Methods in Molecular Biology, Vol. 42), Humana Press, 1995; Challacombe & Kemeny, ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects, John Wiley, 1998; Kemeny, A Practical Guide to ELISA, Pergamon  
20 Press, 1991; Ishikawa, Ultrasensitive and Rapid Enzyme Immunoassay (Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 27), Elsevier, 1991.

Colorimetric Assays for Enzymes - Colorimetry is any method of quantitative chemical analysis in which the concentration or amount of a compound is determined by comparing the color produced by the reaction of a reagent with both standard and test  
25 amounts of the compound, often using a colorimeter. A colorimeter is a device for measuring color intensity or differences in color intensity, either visually or photoelectrically.

Standard colorimetric assays of beta-galactosidase enzymatic activity are well known to those skilled in the art (see, for example, Norton *et al.*, (1985) Mol. Cell. Biol. 5, 281-290). A colorimetric assay can be performed on whole cell lysates using O-nitrophenyl-beta-D-galactopyranoside (ONPG, Sigma) as the substrate in a standard colorimetric beta-galactosidase assay (Sambrook *et al.*, (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Press). Automated colorimetric assays are also available for the detection of beta-galactosidase activity, as described in U.S. Patent No. 5,733,720.

Immunofluorescence Assays - Immunofluorescence or immunofluorescence microscopy is a technique in which an antigen or antibody is made fluorescent by conjugation to a fluorescent dye and then allowed to react with the complementary antibody or antigen in a tissue section or smear. The location of the antigen or antibody can then be determined by observing the fluorescence by microscopy under ultraviolet light.

For general information on immunofluorescent techniques, see, for example, Knapp *et al.*, (1978) Immunofluorescence and Related Staining Techniques, Elsevier; Allan, (1999) Protein Localization by Fluorescent Microscopy: A Practical Approach (The Practical Approach Series, Vol. 218) Oxford University Press; Beutner, (1983) Defined Immunofluorescence and Related Cytochemical Methods, New York Academy of Sciences; Caul, (1993) Immunofluorescence Antigen Detection Techniques in Diagnostic Microbiology, Cambridge University Press. For detailed explanations of immunofluorescent techniques applicable to the present invention, see, U.S. Patent No. 5,912,176; 5,869,264; 5,866,319; and 5,861,259.

Biochips - The peptides of the invention can be used on an array or microarray for high-throughput screening for agents which interact with either the nucleic acids of the invention or their corresponding proteins.

An "array" or "microarray" generally refers to a grid system which has each position or probe cell occupied by a defined nucleic acid fragments also known as

oligonucleotides. The arrays themselves are sometimes referred to as "chips" or "biochips" which are high-density nucleic acid and peptide microarrays often having thousands of probe cells in a variety of grid styles.

A typical molecular detection chip includes a substrate on which an array of recognition sites, binding sites or hybridization sites are arranged. Each site has a respective molecular receptor which binds or hybridizes with a molecule having a predetermined structure. The solid support substrates which can be used to form surface of the array or chip include organic and inorganic substrates, such as glass, polystyrenes, polyimides, silicon dioxide and silicon nitride. For direct attachment of probes to the electrodes, the electrode surface must be fabricated with materials capable of forming conjugates with the probes.

Once the array is fabricated, a sample solution is applied to the molecular detection chip and molecules in the sample bind or hybridize at one or more sites. The sites at which binding occurs are detected, and one or more molecular structures within the sample are subsequently deduced. Detection of labeled batches is a traditional detection strategy and includes radioisotope, fluorescent and biotin labels, but other options are available, including electronic signal transduction.

The methods of this invention will find particular use wherever high through-put of samples is required. In particular, this invention is useful in ligand screening settings and for determining the composition of complex mixtures.

Polypeptides are an exemplary system for exploring the relationship between structure and function in biology. When the twenty naturally occurring amino acids are condensed into a polymeric molecule they form a wide variety of three-dimensional configurations, each resulting from a particular amino acid sequence and solvent condition. For example, the number of possible polypeptide configurations using the twenty naturally occurring amino acids for a polymer five amino acids long is over three million. Typical proteins are more than one-hundred amino acids in length.



In typical applications, a complex solution containing one or more substances to be characterized contacts a polymer array comprising polypeptides. The polypeptides of the invention can be prepared by classical methods known in the art, for example, by using standard solid phase techniques. The standard methods include exclusive solid  
5 phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis and recombinant DNA technology (see Merrifield, (1963) Am. Chem. Soc. 85, 2149-2152).

In a preferred embodiment, the polypeptides or proteins of the array can bind to other co-receptors to form a heteroduplex on the array. In yet another embodiment, the  
10 polypeptides or proteins of the array can bind to peptides or small molecules.

#### D. Uses for Agents That Interact at the NBD

As provided in the Examples, agents that modulate or up- or down-regulate the expression of NEMO or agents such as agonists or antagonists of at least one activity of  
15 NEMO may be used to modulate biological and pathologic processes associated with the NEMO or IKK $\beta$  function and activity. In particular, these agents effect NF- $\kappa$ B mediated processes by interacting with NEMO and can be used to modulate biological or pathological processes associated with NF- $\kappa$ B.

As used herein, a subject can be any mammal, so long as the mammal is in need of  
20 modulation of a pathological or biological process mediated by a protein of the invention. The term "mammal" is meant to identify an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, unregulated expression of NF- $\kappa$ B is associated with pro-  
25 inflammatory processes underlying certain pathological processes. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, pro-inflammatory responses may be prevented or pathological processes modulated by the administration of agents which reduce, promote

or modulate in some way the expression or at least one activity of a NEMO or IKK $\beta$ . Agents can therefore be used to treat diseases with an NF- $\kappa$ B inflammatory component, such disease include but are not limited to; osteoporosis, rheumatoid arthritis, atherosclerosis, asthma (Ray & Cohn, (1999) J. Clin. Invest. 104, 985-993; Christman *et al.*, (2000) Chest 117, 1482-1487) and Alzheimer's disease. For a review of diseases with an NF- $\kappa$ B inflammatory component, see Epstein, (1997) New Eng. J. Med. 336, 1066-1071; Lee *et al.*, (1998) J. Clin. Pharmacol. 38, 981-993; Brand *et al.*, (1997) Exp. Physiol. 82, 297-304.

Pathological processes associated with a pro-inflammatory response in which the agents of the invention would be useful for treatment include, but are not limited to, asthma, allergies such as allergic rhinitis, urticaria, anaphylaxis, drug sensitivity, food sensitivity, etc. and the like; cutaneous inflammation such as dermatitis, eczema, psoriasis, contact dermatitis, sunburn, aging, etc. and the like; arthritis such as osteoarthritis, psoriatic arthritis, lupus, spondylarthritis, etc. and the like. These agents also are useful for treating chronic obstruction pulmonary disease and chronic inflammatory bowel disease. The peptides of the present invention can be used to replace corticosteroids in any application in which corticosteroids are used including immunosuppression in transplants and cancer therapy.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention can be administered in combination with anti-inflammatory agents. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage

administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

#### E. Pharmaceutical Preparations

5           The invention also includes pharmaceutical compositions comprising the agents of the invention together with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical

10           composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Gennaro *et al.*, (1995) Remington's Pharmaceutical Sciences, Mack Publishing Company. In addition to the pharmacologically active agent, the compositions of the present invention may contain

15           suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as

20           appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and dextran. Optionally, the suspension may also

25           contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

          The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all

three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route or by inhalation or lavage, directly to the lungs. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The agents used in the method of treatment of this invention may be administered systemically or topically, depending on such considerations as the condition to be treated, need for site-specific treatment, quantity of drug to be administered and similar considerations.

Topical administration may be used. Any common topical formation such as a solution, suspension, gel, ointment or salve and the like may be employed. Preparation of such topical formulations are well described in the art of pharmaceutical formulations as exemplified, for example, by Remington's Pharmaceutical Sciences. For topical application, these compounds could also be administered as a powder or spray, particularly in aerosol form. The active ingredient may be administered in pharmaceutical compositions adapted for systemic administration. As is known, if a drug is to be administered systemically, it may be confected as a powder, pill, tablet or the like or as a syrup or elixir for oral administration. For intravenous, intraperitoneal or intra-lesional administration, the compound will be prepared as a solution or suspension capable of being administered by injection. In certain cases, it may be useful to formulate these compounds in suppository form or as an extended release formulation for deposit under the skin or intramuscular injection. In a preferred embodiment, the compounds of

this invention may be administered by inhalation. For inhalation therapy the compound may be in a solution useful for administration by metered dose inhalers or in a form suitable for a dry powder inhaler.

An effective amount is that amount which will modulate the activity or alter the level of a target protein. A given effective amount will vary from condition to condition and in certain instances may vary with the severity of the condition being treated and the patient's susceptibility to treatment. Accordingly, a given effective amount will be best determined at the time and place through routine experimentation. However, it is anticipated that in the treatment of a tumor in accordance with the present invention, a formulation containing between 0.001 and 5 percent by weight, preferably about 0.01 to 1 percent, will usually constitute a therapeutically effective amount. When administered systemically, an amount between 0.01 and 100 mg per kg body weight per day, but preferably about 0.1 to 10 mg per kg, will effect a therapeutic result in most instances.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, preferably in humans.

In still another embodiment, the compounds of the invention may be coupled to chemical moieties, including proteins that alter the functions or regulation of target proteins for therapeutic benefit. These proteins may include in combination other inhibitors of cytokines and growth factors that may offer additional therapeutic benefit in the treatment of disorders associated with inflammation. In addition, the molecules of the invention may also be conjugated through phosphorylation to biotinylate, thioate, acetylate, iodinate using any of the cross-linking reagents well known in the art.

#### F. Molecular Biology, Microbiology and Recombinant DNA Techniques

In accordance with the present invention, as described above or as discussed in the Examples below, there may be employed conventional molecular biology, microbiology and recombinant DNA techniques. Such techniques are explained fully in the literature.

5 See for example, Sambrook *et al.*, (1989) *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Press; Glover, (1985) *DNA Cloning: A Practical Approach*; Gait, (1984) *Oligonucleotide Synthesis*; Harlow & Lane, (1988) *Antibodies - A Laboratory Manual*, Cold Spring Harbor Press; Roe *et al.*, (1996) *DNA Isolation and Sequencing: Essential Techniques*, John Wiley; and Ausubel *et al.*, (1995) *Current Protocols in*  
 10 *Molecular Biology*, Greene Publishing.

#### G. Antisense RNA

Antisense molecules are RNA or single-stranded DNA molecules with nucleotide sequences complementary to a specified mRNA. When a laboratory-prepared antisense  
 15 molecule is injected into cells containing the normal mRNA transcribed by a gene under study, the antisense molecule can base-pair with the mRNA, preventing translation of the mRNA into protein. The resulting double-stranded RNA or RNA/DNA is digested by enzymes that specifically attach to such molecules. Therefore, a depletion of the mRNA occurs, blocking the translation of the gene product so that antisense molecules find uses  
 20 in medicine to block the production of deleterious proteins. Methods of producing and utilizing antisense RNA are well known to those of ordinary skill in the art (see, for example, Lichtenstein & Nellen (Editors), *Antisense Technology: A Practical Approach*, Oxford University Press, 1997; Agrawal & Crooke, *Antisense Research and Application* (Handbook of Experimental Pharmacology, Vol. 131), Springer Verlag, 1998; Gibson,  
 25 *Antisense and Ribozyme Methodology: Laboratory Companion*, Chapman & Hall, 1997; Mol & Van Der Krol, *Antisense Nucleic Acids and Proteins*, Marcel Dekker; Weiss, *Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents*, CRC Press, 1997; Stanley *et al.*, (1993) *Antisense Research and*

Applications, CRC Press; Stein & Krieg, (1998) Applied Antisense Oligonucleotide Technology).

Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2'O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept can be extended by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

#### H. Fusion Proteins

A fusion protein is an expression product resulting from the fusion of two genes. Such a protein may be produced, *e.g.*, in recombinant DNA expression studies or, naturally, in certain viral oncogenes in which the oncogene is fused to *gag*.

The production of a fusion protein sometimes results from the need to place a cloned eukaryotic gene under the control of a bacterial promoter for expression in a bacterial system. Sequences of the bacterial system are then frequently expressed linked to the eukaryotic protein. Fusion proteins are used for the analysis of structure, purification, function, and expression of heterologous gene products.

A fused protein is a hybrid protein molecule which can be produced when a nucleic acid of interest is inserted by recombinant DNA techniques into a recipient plasmid and displaces the stop codon for a plasmid gene. The fused protein begins at the amino end with a portion of the plasmid protein sequence and ends with the protein of interest.

The production of fusion proteins is well known to one skilled in the art (See, *e.g.*, U.S. Patent No. 5,908,756; 5,907,085; 5,906,819; 5,905,146; 5,895,813; 5,891,643; 5,891,628; 5,891,432; 5,889,169; 5,889,150; 5,888,981; 5,888,773; 5,886,150; 5,886,149; 5,885,833; 5,885,803; 5,885,779; 5,885,580; 5,883,124; 5,882,941; 5,882,894; 5,882,864; 5,879,917; 5,879,893; 5,876,972; 5,874,304; and 5,874,290). For a general review of the construction, properties, applications and problems associated with specific types of fusion molecules used in clinical and research medicine, see, *e.g.*, Chamow *et al.*, (1999) Antibody Fusion Proteins, John Wiley.

#### I. Peptide Mimetics.

This invention also includes peptide mimetics which mimic the three-dimensional structure of the NBD on IKK $\beta$  and block NEMO binding at the NBD by binding to NEMO. Such peptide mimetics may have significant advantages over naturally-occurring peptides, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others.

In one form, mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, (1993) Peptide Turn Mimetics in Biotechnology and Pharmacy, Pezzuto *et al.*, (Editors) Chapman & Hall. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate



molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

In another form, peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide.

5 These types of non-peptide compounds are also referred to as "peptide mimetics" or "peptidomimetics" (Fauchere, (1986) *Adv. Drug Res.* 15, 29-69; Veber & Freidinger, (1985) *Trends Neurosci.* 8, 392-396; and Evans *et al.*, (1987) *J. Med. Chem.* 30, 1229-1239, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling.

10 Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptide mimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biochemical property or pharmacological activity), such as the NBD, but have one or more peptide linkages optionally replaced by a linkage selected from the group  
 15 consisting of:  $-\text{CH}_2\text{NH}-$ ,  $-\text{CH}_2\text{S}-$ ,  $-\text{CH}_2\text{CH}_2-$ ,  $-\text{CH}=\text{CH}-$  (cis and trans),  $-\text{COCH}_2-$ ,  $-\text{CH}(\text{OH})\text{CH}_2-$ , and  $-\text{CH}_2\text{SO}-$ , by methods known in the art and further described in the following references: Weinstein, (1983) *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Marcel Dekker; Morley, (1980) *Trends Pharmacol. Sci.* 1, 463-468 (general review); Hudson *et al.*, (1979) *Int. J. Pept. Protein Res.* 14, 177-185 ( $-\text{CH}_2\text{NH}-$ ,  
 20  $\text{CH}_2\text{CH}_2-$ ); Spatola *et al.*, (1986) *Life Sci.* 38, 1243-1249 ( $-\text{CH}_2\text{S}-$ ); Hann, (1982) *J. Chem. Soc. Perkin Trans.* 1, 307-314 ( $-\text{CH}-\text{CH}-$ , cis and trans); Almquist *et al.*, (1980) *J. Med. Chem.* 23, 1392-1398 ( $-\text{COCH}_2-$ ); Jennings-White *et al.*, (1982) *Tetrahedron Lett.* 23, 2533 ( $-\text{COCH}_2-$ ); U.S. Patent Application No. 4,424,207 ( $-\text{CH}(\text{OH})\text{CH}_2-$ ); Holladay *et al.*, (1983) *Tetrahedron Lett.* 24, 4401-4404 ( $-\text{C}(\text{OH})\text{CH}_2-$ ); and Hruby, (1982) *Life Sci.*  
 25 31, 189-199 ( $-\text{CH}_2\text{S}-$ ); each of which is incorporated herein by reference.

Labeling of peptide mimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptide mimetic that are predicted by quantitative structure-activity data and/or

molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (*e.g.*, are not contact points in NBD-NEMO complexes) to which the peptide mimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptide mimetics should not substantially interfere with the desired biological or pharmacological activity of the peptide mimetic.

NBD peptide mimetics can be constructed by structure-based drug design through replacement of amino acids by organic moieties (see, for example, Hughes, (1980) *Philos. Trans. R. Soc. Lond.* 290, 387-394; Hodgson, (1991) *Biotechnol.* 9, 19-21; Suckling, (1991) *Sci. Prog.* 75, 323-359).

The use of peptide mimetics can be enhanced through the use of combinatorial chemistry to create drug libraries. The design of peptide mimetics can be aided by identifying amino acid mutations that increase or decrease binding of NEMO at the NBD on IKK $\beta$ . For example, such mutations as identified in Table 1. Approaches that can be used include the yeast two hybrid method (see Chien *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88, 9578-9582) and using the phage display method. The two hybrid method detects protein-protein interactions in yeast (Fields *et al.*, (1989) *Nature* 340, 245-246). The phage display method detects the interaction between an immobilized protein and a protein that is expressed on the surface of phages such as lambda and M13 (Amberg *et al.*, (1993) *Strategies* 6, 2-4; Hogrefe *et al.*, (1993) *Gene* 128, 119-126). These methods allow positive and negative selection for protein-protein interactions and the identification of the sequences that determine these interactions.

For general information on peptide synthesis and peptide mimetics, see, for example, Jones, (1992) *Amino Acid and Peptide Synthesis*, Oxford University Press; Jung, (1997) *Combinatorial Peptide and Nonpeptide Libraries: A Handbook*, John Wiley; and Bodanszky *et al.*, (1993) *Peptide Chemistry: A Practical Textbook*, 2nd Revised Edition, Springer Verlag each of which is hereby incorporated in its entirety.

### J. Transgenic Animals

Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to as a transgene. The nucleic acid sequence of the transgene may be integrated either at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability of the transgenic animal to transfer the genetic information to offspring. If such offspring in fact possess some or all of that alteration or genetic information, then they too are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or may be genetic information already possessed by the recipient. In the last case, the altered or introduced gene may be expressed differently than the native gene.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (see, *e.g.*, U.S. Patent No. 4,736,866; U.S. Patent No. 5,602,307; Mullins *et al.*, (1993) Hypertension 22, 630-633; Brenin *et al.*, (1997) Surg. Oncol. 6, 99-110; Tuan, (1997) Recombinant Gene Expression Protocols, Methods in Molecular Biology No. 62, Humana Press).

A number of recombinant or transgenic mice have been produced, including those which express an activated oncogene sequence (U.S. Patent No. 4,736,866); express simian SV40 T-antigen (U.S. Patent No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent No. 5,723,719); express at least one human gene which

participates in blood pressure control (U.S. Patent No. 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent No. 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent No. 5,602,307); possess a bovine growth hormone gene (Clutter *et al.*, (1996) Genetics 143, 1753-1760) or, are capable of generating a fully human antibody response (Zou *et al.*, (1993) Science 262, 1271-1274).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (see Kim *et al.*, (1997) Mol. Reprod. Dev. 46, 515-526; Houdebine, (1995) Reprod. Nutr. Dev. 35, 609-617; Petters, (1994) Reprod. Fertil. Dev. 6, 643-645; Schnieke *et al.*, (1997) Science 278, 2130-2133; Amoah, (1997) J. Animal Science 75, 578-585).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Patent No. 5,489,743 and U.S. Patent No. 5,602,307.

The present invention comprises transgenic animals expressing a gene encoding the NBD, and mutations of that gene resulting in conservative and non-conservative amino acid substitutions when compared to the wild type gene.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

## EXAMPLES

### Example 1 - Identification of NEMO binding domain on IKK $\beta$

To identify the NEMO-interacting domain of IKK $\beta$  we performed *in vitro* pull down assays (Zhong *et al.*, (1997) Cell 89, 413-424) using a bacterially expressed version of full length NEMO fused at its NH<sub>2</sub>-terminus to glutathione S-transferase (GST-NEMO; Figure 1A). Various truncation mutants lacking different functional domains of IKK $\beta$  (catalytic domain, leucine zipper and helix-loop-helix; Figure 1A) were constructed.

All sub-cloning and mutagenesis of full length cDNA clones of IKK $\alpha$  and IKK $\beta$  was performed by PCR using cloned Pfu DNA-polymerase (Stratagene). The wild-type and mutated IKK $\beta$  cDNA were inserted into the *KpnI* and *NotI* restriction sites of pcDNA-3 or pcDNA-3.1-xpress (Invitrogen) and all IKK $\alpha$  cDNAs were inserted into the *EcoRI* and *XhoI* sites of the same vectors. FLAG-tagged versions of both kinases were constructed by subcloning into pFLAG-CMV-2 (Sigma). For GST-IKK $\beta$ -(644-756), the PCR fragment was inserted into the *EcoRI* and *XhoI* sites of pGEX-4T1 (Pharmacia).

Full length cDNA encoding human NEMO was obtained by reverse transcriptase (RT)-PCR from HeLa cell mRNA using the Expand<sup>TM</sup> Long Template PCR System (Boehringer Mannheim) and the primer pair (5'-ATAGACGAATTCAATAGGCACCTCTGGAAG) (SEQ ID NO: 20) and (3'-TAGGACCTCGAGCTACTCAATGCACTCCATG) (SEQ ID NO: 21). The resulting PCR fragment was inserted into the *EcoRI* and *XhoI* sites of pcDNA-3 or pcDNA-s.1-xpress. All subsequent NEMO mutants were constructed by PCR using Pfu DNA-polymerase. GST-NEMO was constructed by sub-cloning the full-length cDNA into the *EcoRI* and *XhoI* sites of pGEX-4T1.

These mutants were labeled by *in vitro* translation with [<sup>35</sup>S]-methionine (input; Figure 1B), mixed with either GST alone or GST-NEMO, and precipitated using glutathione-agarose. None of the mutants interacted with GST alone, whereas wild-type and all three NH<sub>2</sub>-terminal truncations of IKK $\beta$  (307-756, 458-756 and 486-756) interacted with GST-NEMO (Figure 1B (right panel). In contrast, none of the COOH-

terminal truncation mutants (1-456, 1-605 or 1-644) precipitated with GST-NEMO. These results demonstrate that NEMO interacts with a region in the COOH-terminus of IKK $\beta$  distal to the helix-loop-helix (HLH) domain. A mutant consisting of only the region from amino acid 644 (immediately after the HLH) to the COOH-terminus (residue 756) of  
5 IKK $\beta$  was constructed next. As shown in Figure 1C, this mutant did not precipitate with GST but did interact with GST-NEMO confirming that this region mediates the interaction between these two molecules.

The effects of IKK $\beta$ -(644-756) on IL-1 $\beta$ - and TNF $\alpha$ -induced NF- $\kappa$ B activation by transiently transfecting HeLa cells with the mutant together with an NF- $\kappa$ B-dependent  
10 reporter plasmid (pBIIIX-luciferase) was tested next (Kopp & Ghosh, (1994) Science 265, 956-959). For transfection studies, HeLa and COS cells were seeded into either twenty-four well (1 $\times$ 10<sup>5</sup> cells/well) or six well (5 $\times$ 10<sup>5</sup> cells/well) plates and grown for twenty-four hours before transfection of DNA with Fugene6 (Roche) according to the manufacturer's protocol. Cells in twenty-four well and six well trays received a total of 1  
15  $\mu$ g or 2  $\mu$ g of DNA respectively. After forty-eight hours cells were lysed with TNT (200 mM NaCl, 20 mM Tris-pH 8.0, 1% Triton-100) and the lysate were used for either immunoprecipitation or luciferase assay (Primage Luciferase Assay System).

Figure 1D shows that IKK $\beta$ -(644-756) inhibited NF- $\kappa$ B activation induced by these cytokines in a dose-dependent manner. These results indicate that IKK $\beta$ -(644-756)  
20 acts as a dominant-negative by titrating endogenous NEMO out of the core I $\kappa$ B-kinase complex. Without the recruitment of regulatory proteins by NEMO, IKK $\beta$  becomes refractory to IL-1 $\beta$ - and TNF $\alpha$ -induced signals that should otherwise cause its activation.

Structurally, NEMO consists of extensive  $\alpha$ -helical regions containing two prominent stretches of coiled-coil and a leucine-zipper motif, and a COOH-terminal zinc-  
25 finger domain (Figure 2A) (Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Yamaoka *et al.*, (1998) Cell 93, 1231-1240; Rothwarf *et al.*, (1998) Nature 395, 297-300). Previous studies attempting to identify the region of NEMO required for its interaction with IKK $\beta$  have generated conflicting results (Harhaj *et al.*, (1999) J. Biol. Chem. 274,

15297-15300). To address this question GST-pull-down assays using a GST-fusion protein of IKK $\beta$ -(644-756) (Figure 2A) and various [ $^{35}$ S]-methionine-labeled truncation mutants of NEMO (Figure 2A) were performed. Figure 2A (right panel) summarizes the results of these experiments in which it was demonstrated that IKK $\beta$ -(644-756) interacted with NEMO-(1-196), -(1-302) and -(44-419) but not NEMO-(197-419) or -(86-419). Identical results were obtained from immuno-precipitation studies using lysate of COS or HEK293 cells transiently transfected with FLAG-tagged IKK $\beta$  and the NEMO mutants (data not shown).

For all immunoprecipitations HeLa or COS cells grown in six well trays were lysed in 500  $\mu$ l TNT. FLAG-tagged proteins were precipitated from lysate of transfected cells for two hours at 4°C using 20  $\mu$ l of anti-FLAG (M2)-conjugated agarose beads (Sigma). Immunoprecipitations of endogenous IKK $\beta$  or NEMO were performed using 1  $\mu$ g of specific rabbit polyclonal antibodies (Santa Cruz) plus 20  $\mu$ l of Protein-A sepharose (Amersham-Pharmacia). For immunoblotting, precipitates were washed three times with TNT, twice with PBS then suspended in SDS-sample buffer. Proteins were separated by SDS-PAGE (10%), transferred to PVDF membranes and visualized by enhanced chemiluminescence (Amersham-Pharmacia).

These results establish that the interaction domain lies between residues 44 and 86, a region including the first  $\alpha$ -helix of NEMO. A mutant was therefore made in which  $\alpha$ -helix up to the first coiled-coil domain was deleted (residues T50-L93; del. $\alpha$ H). This mutant did not interact with IKK $\beta$ -(644-756) (Figure 2B). Furthermore transfection studies using pBIIIX-luciferase demonstrated that del. $\alpha$ H inhibited TNF $\alpha$ -induced NF- $\kappa$ B activity (Figure 2C) confirming previous reports that the COOH-terminus of NEMO which can not interact with IKK $\beta$ , is a dominant-negative inhibitor of NF- $\kappa$ B (Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Rothwarf *et al.*, (1998) Nature 395, 297-300). Taken together, Figures 1 and 2 show that the interaction between IKK $\beta$  and NEMO occurs via the COOH-terminus of IKK $\beta$  and the first  $\alpha$ -helical region of NEMO. These findings suggest a model in which the NH $_2$ -terminus of NEMO anchors it to the

IKK-complex leaving the remainder of the molecule containing several protein:protein interaction domains free and accessible for interacting with upstream regulators of IKK function.

5 Example 2 - NEMO regulation of IKK $\beta$  function through interaction at NBD

To fully characterize the NEMO-interaction domain of IKK $\beta$  further truncation mutants between residues V644 and S756 (Figure 3A) were constructed. Immediately after the HLH, the amino acid sequence to the cysteine at position 662 exhibits 72% identity with IKK $\alpha$  (denoted  $\alpha_1$  in Figure 3A). Following this, the region up to E707 is a serine-rich domain previously reported to be a target for auto-phosphorylation and to function in down-regulating IKK $\beta$  activity after stimulation by pro-inflammatory cytokines (Delhase *et al.*, (1999) Science 284, 309-313). The sequence succeeding this contains no serine residues until position 733. Mutants sequentially omitting each of these regions were [<sup>35</sup>S]-methionine-labeled and used in GST-pull-down assays as described above. Figure 3A summarizes the results from these experiments demonstrating that none of the IKK $\beta$  mutants precipitated with GST-NEMO and indicating that the interaction domain resides in the extreme COOH-terminus between residues F734 and S756.

Comparison of this short segment of IKK $\beta$  with the corresponding region of IKK $\alpha$  reveals two striking structural characteristics (Figure 3B). First, the sequence from F734 to T744 of IKK $\beta$  ( $\alpha_2$  in Figure 3B) is identical to the equivalent sequence in IKK $\alpha$  (L737 to L742 of IKK $\beta$  and L738 to L743 of IKK $\alpha$ ). Second, the sequence of IKK $\beta$  extends beyond the COOH-terminal residue of IKK $\alpha$  (E745) for twelve amino acids comprising a highly acidic region in which five of the residues are glutamates (Figure 3B). The marked similarity between the  $\alpha_2$ -region of IKK $\beta$  and the extreme COOH-terminus of IKK $\alpha$  together with previous reports that NEMO does not interact with IKK $\alpha$  *in vitro* (Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Yamaoka *et al.*, (1998) Cell 93, 1231-1240; Rothwarf *et al.*, (1998) Nature 395, 297-300) led to the hypothesis that the NEMO-



interaction domain would be the glutamate-rich portion of IKK $\beta$  (E745 to S756). To test this hypothesis, a truncation mutant omitting this region was made (1-744; Figure 3C) and investigated for its ability to interact with GST-NEMO. The mutant associated with GST-NEMO to an equal extent as wild-type IKK $\beta$  (Figure 3C); these results have been confirmed by co-immunoprecipitating epitope-tagged NEMO and IKK $\beta$ -(1-744) from lysate of transiently transfected COS cells. These findings demonstrate that the NEMO-interaction domain of IKK $\beta$  is within the  $\alpha$ <sub>2</sub>-region of the COOH-terminus.

IKK $\beta$  COOH-terminal truncation mutants were next used to test the effects of NEMO association on basal and induced activity of IKK $\beta$ : Figure 3D shows that truncation of IKK $\beta$  at V644, eliminating the serine-rich region (see Figure 3A), resulted in complete loss of basal auto-phosphorylation. In contrast, a mutant containing the serine-rich region (1-733), exhibited significantly higher levels of auto-phosphorylation than wild-type IKK $\beta$  (Figure 3D). Intriguingly, the level of auto-phosphorylation of IKK $\beta$ -(1-744) which contains the NEMO-binding  $\alpha$ <sub>2</sub>-region, was identical to that observed with the wild-type kinase. To test the effects that these mutations have on basal kinase activity, mutants were transiently transfected into HeLa cells and NF- $\kappa$ B activity determined by luciferase assay as described in Example 1. The results in Figure 3D demonstrate that IKK $\beta$ -(1-644) did not induce NF- $\kappa$ B activity whereas IKK $\beta$ -(1-733) caused increased activation compared with wild-type (Figure 3E). Furthermore, NF- $\kappa$ B activity induced by IKK $\beta$ -(1-744) was identical to that induced by wild-type IKK $\beta$ . These results demonstrate that basal auto-phosphorylation and kinase activity of IKK $\beta$  is dependent on the ability of NEMO to associate with the kinase. One explanation for these observations may be that NEMO recruits a phosphatase to the IKK-complex that regulates basal IKK $\beta$  function by targeting the serine-rich region of the COOH-terminus. Inability to bind NEMO therefore prevents phosphatase recruitment and causes increased phosphorylation within this region.

To directly test the effect that loss of the  $\alpha$ <sub>2</sub>-region has on the catalytic activity of IKK $\beta$ , an immune-complex kinase assay was performed on lysate from transfected HeLa

cells (Figure 3F). For immune-complex kinases assays, precipitates were washed with TNT then with kinase buffer (20 mM HEPES pH 7.5, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM NaF, 2 mM  $\beta$ -glycerophosphate, 1 mM DTT, 10  $\mu$ M ATP). Precipitates were then incubated for fifteen minutes at 30°C in 20  $\mu$ l of kinase buffer containing GST-I $\kappa$ B $\alpha$ -(1-90) and 10  $\mu$ Ci [<sup>32</sup>P]- $\gamma$ -labeled ATP (Amersham-Pharmacia). The substrate was precipitated using glutathione-agarose (Amersham-Pharmacia) and separated by SDS-PAGE (10%). Kinase activity was determined by autoradiography. Phosphorylated proteins associated with the kinase complex appeared on autoradiographs because the immuno-precipitated complex was not removed prior to GST- substrate precipitation.

Activity of IKK $\beta$  (wild-type) was low in untreated cells but was markedly enhanced after treatment with TNF $\alpha$ . Consistent with the data presented in Figure 3E, basal activity of IKK $\beta$ -(1-733) was significantly higher than wild-type, however this activity was not further enhanced by treatment with TNF $\alpha$  (Figure 3F). Furthermore, basal and TNF $\alpha$ -induced catalytic activity of IKK $\beta$ -(1-744) was identical to the activity of IKK $\beta$  (WT). In addition to phosphorylated GST-I $\kappa$ B $\alpha$ , auto-phosphorylated IKK $\beta$  proteins were also detected (Figure 3F, top bands). Following TNF $\alpha$  treatment, IKK $\beta$  (WT) and IKK $\beta$ -(1-744) became rapidly autophosphorylated whereas the already high basal phosphorylation of IKK $\beta$ -(1-733) was only slightly enhanced (Figure 3F). A previous study showed that auto-phosphorylation serves to down-regulate TNF $\alpha$ -induced IKK $\beta$  activity by causing conformational changes within the protein (Delhase *et al.*, (1999) Science 284, 309-313). Taken together, these findings (Figures 3D-F) demonstrate that in the absence of NEMO, IKK $\beta$  becomes auto-phosphorylated, basally active and refractory to TNF $\alpha$ -induced signals indicating that NEMO plays a fundamental role in the down-regulation as well as activation of IKK $\beta$  activity.

An additional band representing a phosphorylated protein appeared only in the samples from TNF $\alpha$ -induced IKK $\beta$  (WT) and IKK $\beta$ -(1-744) transfected cells (Figure 3F). The molecular weight of this protein (49 kDa) strongly suggests that it is endogenous NEMO associated with the precipitated complex. This is supported by the absence of the

band in either precipitate (+/- TNF $\alpha$ ) from IKK $\beta$ -(1-733) transfected cells. This protein has been identified as phosphorylated NEMO by dissociating the precipitated complex in SDS and re-immunoprecipitating [<sup>32</sup>P]-labeled NEMO using specific anti-NEMO antibodies. Induced phosphorylation of NEMO may therefore represent a further level of regulation of the activity of the IKK complex.

Example 3 - Identification of the NBD on IKK $\alpha$

Since the  $\alpha_2$ -region of IKK $\beta$  strongly resembles the COOH-terminus of IKK $\alpha$  (Figure 3B), the ability of IKK $\alpha$  to interact with NEMO was tested.

Immunoprecipitations from lysate of COS cells transiently transfected with xpress-tagged NEMO together with FLAG-tagged versions of either IKK $\alpha$  or IKK $\beta$  were performed using anti-FLAG as described in Example 1. Figure 4A shows that NEMO interacted equally well with both IKK $\beta$  and IKK $\alpha$ . It is possible that in this experiment the interaction with IKK $\alpha$  is not direct but due instead to the formation of a complex containing endogenous IKK $\beta$ , FLAG-IKK $\alpha$  and xpress-NEMO. A GST-pull-down assays was therefore performed using GST-NEMO and [<sup>35</sup>S]-methionine-labeled versions of either wild-type IKK $\alpha$  or a truncated IKK $\alpha$  mutant lacking the eight COOH-terminal amino acids (1-737: Figure 4B). In agreement with the findings presented above (Figure 4A), but in contrast to previous reports (Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Yamaoka *et al.*, (1998) Cell 93, 1231-1240; Rothwarf *et al.*, (1998) Nature 395, 297-300), wild-type IKK $\alpha$  interacted with NEMO *in vitro* whereas the truncated mutant did not (Figure 4B). These results not only demonstrate that IKK $\alpha$  interacts with NEMO but also shows that it does so via the COOH-terminal region containing the six amino acids shared between IKK $\alpha$  and the  $\alpha_2$ -region of IKK $\beta$  (Figure 3B). Gene-targeting studies have demonstrated profound differences in the activation of IKK $\alpha$  and IKK $\beta$  by TNF $\alpha$  (Woronicz *et al.*, (1997) Science 278, 866-869; Zandi *et al.*, (1997) Cell 91, 243-252; Mercurio *et al.*, (1997) Science 278, 860-866; DiDonato *et al.*, (1997) Nature 388, 548-554; Régnier *et al.*, (1997) Cell 90, 373-383).

002280" 09224960

The present findings indicate that the basis of this difference is not due to differential recruitment of NEMO (Delhase *et al.*, (1999) Science 284, 309-313; Takeda *et al.*, (1999) Science 284, 313-316; Hu *et al.*, (1999) Science 284, 316-20; Li *et al.*, (1999) Science 284, 321-325; Li *et al.*, (1999) J. Exp. Med. 189, 1839-1845; Li *et al.*, (1999) Genes Dev. 13, 1322-1328; Tanaka *et al.*, (1999) Immunity 10, 421-429). Instead the difference most likely lies in the ability of each kinase to integrate NEMO-associated signaling components into an activation response, presumably through differences in the inherent regulatory features of the individual kinases.

Further evidence that this short COOH-terminal sequence constitutes the NEMO-interaction domain of the IKKs was obtained when we tested the ability of the recently described IKK-related kinase IKK $\epsilon$  (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362) to interact with NEMO. Sequence comparison with IKK $\alpha$  and IKK $\beta$  (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362; Woronicz *et al.*, (1997) Science 278, 866-869; Zandi *et al.*, (1997) Cell 91, 243-52; Mercurio *et al.*, (1997) Science 278, 860-866; DiDonato *et al.*, (1997) Nature 388, 548-554; Régnier *et al.*, (1997) Cell 90, 373-383) reveals that IKK $\epsilon$  does not contain the  $\alpha_2$ -region in its COOH-terminus (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362) and consistent with this being the NEMO binding domain we found that IKK $\epsilon$  does not interact with GST-NEMO in pull down assays (Figure 4C). This finding indicates that NEMO is not required for the functional activity of IKK $\epsilon$  and this is supported by the inability of IKK $\epsilon$  to respond to signals induced by either TNF $\alpha$  or IL-1 $\beta$  (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362).

#### Example 4 - Mutation of amino acid residues in the NBD

Having determined that the  $\alpha_2$ -region of IKK $\beta$ , and the equivalent six amino acid sequence of IKK $\alpha$  are critical for interaction with NEMO [designated NEMO binding domain (NBD)], a deletion mutant in IKK $\beta$  lacking the six amino acids from L737 to L742 (del.NBD) was constructed. This deletion mutant did not associate with GST-NEMO (Figure 4D). Examination of predicted structural and biochemical features of the

NBD in context with surrounding residues suggests that it constitutes an inflexible hydrophobic “pocket” within a hydrophilic region of the IKK $\beta$  COOH-terminus (Figure 4E). This suggests a model in which the NBD becomes buried within the first  $\alpha$ -helical region of bound NEMO (Figure 2) preventing its exposure to an aqueous environment thereby maintaining a strong inter-molecular interaction. Whether the interaction is indeed a function of this hydrophobicity remains to be determined, however we found that substitution of either W739 or W741 with alanine prevented association of NEMO with IKK $\beta$  (Figure 4F). Therefore each of these hydrophobic tryptophan residues is critical for maintaining a functional NBD. In addition, mutation of D738 to alanine also prevented NEMO interaction indicating that a negatively charged residue at this position is required for NBD function. In contrast to these mutations, substitution of L737, S740 or L742 with alanine did not affect NEMO binding. To test the effects of these alanine substitutions on IKK $\beta$  function, HeLa cells were co-transfected with each of the point mutants together with pBIIX-luciferase reporter. Consistent with the observation that the basal activity of IKK $\beta$  is increased in the absence of associated NEMO, IKK $\beta$ -(1-733) (Figure 3E), mutants that did not bind NEMO (D738A, W739A and W741A) activated NF- $\kappa$ B to a greater extent than wild-type IKK $\beta$  or IKK $\beta$ -(1-744) (Figure 4G). In contrast, mutants containing substitutions that did not disrupt NEMO association (L737A, S740A and L742A) induced NF- $\kappa$ B to the same level as the controls. These results indicate that NEMO plays a critical role in the down-regulation of intrinsic IKK $\beta$  activity.

Further mutations in the NBD were analyzed (see Table 1) for their ability to affect NEMO binding to IKK $\beta$  using the GST pulldown assay explained in Example 3.

**Table 1. Characterized NBD mutants and their ability to bind to NEMO.**

<b>NBD Mutants<sup>†</sup></b>	<b>Binds to NEMO</b>	<b>SEQ ID NO:</b>
LDWSWL	yes	2
LDASAL	no	3
ADWSWL	yes	4
LDWSWA	yes	5
<sup>237</sup> <b>ADWSWA</b> <sup>712</sup>	yes ✓	6 ✓
LAWSWL	no	7
LEWSWL	yes	8
LNWSWL	yes	9
LDASWL	no	10
LDFSWL	yes	11
LDYSWL	yes	12
LDWSAL	no	13
LDWSFL	no	14
LDWSYL	no	15
LDWAWL	yes	16
LDWEWL	yes	17

<sup>†</sup> The substituted amino acid residue is indicated by bold face.

**Example 5 - Agents which interact with NBD to block NEMO binding**

The relatively small size of the NBD makes it an attractive target for the development of compounds aimed at disrupting the core IKK complex. The relevance of this approach was investigated by designing cell-permeable peptides spanning the IKK $\beta$  NBD and determining their ability to dissociate the IKK $\beta$ -NEMO interaction.

The sequences of the two NBD peptides used in this study were [DRQIKIWFQNRRMKWKK]TALDWSWLQTE (wild-type) (SEQ ID NO: 18) and [DRQIKIWFQNRRMKWKK]TALDASALQTE (mutant) (SEQ ID NO: 19). The *antennapedia* homeodomain sequence (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450; U.S. Patent No. 5,888,762; U.S. Patent No. 6,015,787; U.S. Patent No. 6,080,724) is bracketed and the positions of the W→A mutations are underlined. Both peptides were dissolved in DMSO to a stock concentration of 20 mM. For all experiments DMSO alone controls were no different from no peptide controls.

The wild-type NBD peptide consisted of the region from T735 to E745 of IKKβ fused with a sequence derived from the third helix of the antennapedia homeodomain that has been shown to mediate membrane translocation (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450). The mutant was identical except that the tryptophan residues (W739 and W741) in the NBD were mutated to alanine. Figure 5A shows that the NBD (WT) but not the mutant dose-dependently inhibited *in vitro* pull-down of [<sup>35</sup>S]-labeled IKKβ by GST-NEMO and [<sup>35</sup>S]-labeled NEMO by GST-IKKβ-(644-756). To test the ability of the NBD peptides to enter cells and inhibit the IKKβ-NEMO interaction, HeLa cells were incubated with the peptides for different time periods and immunoprecipitated the IKK complex using anti-NEMO. In agreement with the *in vitro* data (Figure 5A), wild-type but not mutant disrupted the formation of the endogenous IKK complex (Figure 5B).

#### Example 6 - Agents which block NEMO function

The effects of the NBD peptides on signal-induced activation of NF-κB were investigated next. After transfecting HeLa cells with the pBIIX-luciferase reporter, cells were preincubated with wild-type or mutant peptides, treated with TNFα and NF-κB activation measured by the luciferase reporter assay. As shown in Figure 5C (left panel), the wild-type NBD peptide inhibited TNFα-induced NF-κB activation whereas the mutant had no effect. Interestingly, the basal NF-κB activity was enhanced by treatment with the wild-type peptide (Figure 5C; right panel), a finding which concurs with results

from previous mutational analysis (Figures 3E-F and 4G). This indicates that removal of NEMO increases the basal, intrinsic activity of IKK, while abolishing its responsiveness to TNF $\alpha$ . Further analysis using electrophoretic mobility shift assays (EMSA) also demonstrated that only the wild-type NBD peptide inhibited TNF $\alpha$ -induced activation and nuclear translocation of NF- $\kappa$ B (Figure 5D). Taken together these results demonstrate that delivery of an intact NBD peptide into cells disrupts the IKK $\beta$ -NEMO interaction and prevents pro-inflammatory signals from activating NF- $\kappa$ B. In contrast, transduction with a peptide containing mutations at the tryptophan residues that are critical for maintaining the NEMO interaction has no effect.

#### Example 7 - Agents capable of down-regulating E-selectin

Many proteins involved in the initiation and maintenance of inflammatory responses require NF- $\kappa$ B activation for induced expression of their genes (Ghosh *et al.*, (1998) Annu. Rev. Immunol. 16, 225-260; May & Ghosh, (1998) Immunol. Today 19, 80-88). One such protein, E-selectin, is a leukocyte adhesion molecule expressed on the luminal surface of vascular endothelial cells after activation by pro-inflammatory stimuli such as IL-1 or TNF $\alpha$  (Pober *et al.*, (1986) J. Immunol. 436, 1680-1687; Bevilacqua *et al.*, (1987) Proc. Natl. Acad. Sci. USA 84, 9238-9242; Collins *et al.*, (1995) FASEB J. 9, 899-909). Expression of E-selectin and other NF- $\kappa$ B-dependent adhesion molecules is crucial for the arrest and recruitment of leukocytes into sites of acute and chronic inflammation. To assess the anti-inflammatory potential of the NBD peptide, primary human umbilical vein endothelial cells (HUVEC) were pretreated with the wild-type and mutant peptides and E-selectin expression induced with TNF $\alpha$ . Consistent with the effects on basal NF- $\kappa$ B activation (Figure 5C), the wild-type NBD peptide induced low level expression of E-selectin (Figure 5E). However, after TNF $\alpha$ -treatment the wild-type but not mutant significantly reduced expression of E-selectin (Figure 5E). Inhibition by wild-type NBD peptide reduced expression to the level induced by the peptide in the absence of TNF $\alpha$ .



The importance of the present invention can be viewed on two levels. First, Applicants have identified the structural requirements for the association of NEMO with the IKKs and found that association with IKK $\beta$  is dependent on three amino acids (D738, W739 and W741) within the NBD. Furthermore, NEMO not only functions in the

5 activation of IKK $\beta$  but it also has a critical role in suppressing the intrinsic, basal activity of the IKK complex. The second level of importance is the obvious clinical use for drugs targeting the NBD. Applicants have demonstrated that a cell-permeable peptide encompassing the NBD is able to not only inhibit TNF $\alpha$ -induced NF- $\kappa$ B activation but also reduce expression of E-selectin, an NF- $\kappa$ B-dependent target gene, in primary human

10 endothelial cells. The NBD is only six amino acids long, and therefore it is within the ability of one skilled in the art to design peptido-mimetic compounds that disrupt the core IKK complex. Since the effect of disrupting the complex is to increase the basal activity of the IKK, treatment with an NBD-targeting compound can avoid issues of toxicity, *e.g.*, due to hepatocyte apoptosis, that might arise from administering drugs that completely

15 abolish the activity of NF- $\kappa$ B. Hence, identification of the NBD is a means for the development of novel anti-inflammatory drugs that prevent activating signals from reaching the IKK complex, yet maintain a low level of NF- $\kappa$ B activity and avoid potential toxic side-effects.

20 Example 8 - NBD peptide-mediated inhibition of inflammatory response *in vivo*

The NBD peptide was tested for its ability to inhibit inflammatory responses in animals using two distinct models of acute inflammation. In the first model, ear edema was induced in mice using phorbol-12-myristate-13-acetate (PMA) and the effects of topical administration of the NBD peptides were measured. Ear edema using PMA was

25 induced in replicate groups of age and sex matched mice as previously described (Chang *et al.*, (1987) Eur. J. Pharmacol. 142, 197-205). Twenty  $\mu$ l of either NBD peptides (200  $\mu$ g/ear), dexamethasone (40  $\mu$ g/ear) or vehicle (DMSO:Ethanol; 25:75 v/v) was applied topically to the right ear of mice thirty minutes before and thirty minutes after the

application of 20  $\mu$ l of PMA (5  $\mu$ g/ear) dissolved in ethanol. Ear swelling was measured six hours after PMA application using a microgauge and expressed as the mean difference in thickness between the treated (right) and untreated (left) ears. Statistical analysis of the data was performed using the students t-test. A value of  $p < 0.05$  was considered statistically significant.

Figure 6A shows that the wild type peptide significantly reduced ( $77 \pm 3\%$  inhibition;  $p < 0.05$ ) PMA-induced ear thickening to the level observed with dexamethasone ( $82 \pm 9\%$  inhibition;  $p < 0.05$ ). In contrast, the effect observed with an equivalent dose of mutant was insignificant ( $p = 0.09$ ). Neither peptide had an effect when administered in the absence of PMA (not shown).

In a second model, peritonitis was induced in mice by intraperitoneal (i.p.) injection of zymosan either alone or in combination with dexamethasone or the NBD peptides. For zymosan-induced peritonitis, measurement of peritoneal exudates and inflammatory cell collections from replicate groups of age and sex matched mice (C57BL/6NCR) were performed as previously described (Getting *et al.*, (1998) Immunology 95, 625-630). Groups of animals were injected concomitantly with one ml zymosan (1 mg/ml) and either dexamethasone (100 mg/ml) or the NBD peptides (200 mg/ml). The concentration of NOX (nitrate plus nitrite) present in the inflammatory exudates was measured using a colorimetric assay kit (Alexis Corporation) according to the manufacturers protocol.

As shown in Figure 6B zymosan injection caused an accumulation of inflammatory exudate fluids and migration of polymorphonuclear cells (PMN) into the peritoneum of these animals. Treatment of mice with wild type NBD peptide or dexamethasone significantly reduced exudate formation and PMN accumulation whereas the mutant had no effect.

Various *in vivo* studies have demonstrated a role for NO in exudate formation and leukocyte migration into inflammatory sites (Ialenti *et al.*, (1992) Eur. J. Pharmacol. 211, 177-182; Ialenti *et al.*, (1993) Br. J. Pharmacol. 110, 701-706; Iuvone *et al.*, (1998) Br. J.

Pharmacol. 123, 1325-1330). Therefore the effects of the NBD peptides on NOX accumulation in the peritoneal exudates of zymosan-treated mice was investigated. Figure 6C (lower panel) shows that dexamethasone and wild-type peptide reduced NOX by  $86 \pm 7\%$  and  $66 \pm 4\%$  respectively whereas the mutant had no effect. These results are consistent with previous studies demonstrating that reduction of exudate formation and cell accumulation closely correlate with inhibition of NF- $\kappa$ B activation and reduction of NO formation (D'Acquisto *et al.*, (1999) Eur. J. Pharmacol. 369, 223-236; D'Acquisto *et al.*, (1999) Naunyn-Schmeideberg's Arch. Pharmacol. 360, 670-675). Therefore the wild-type NBD peptide is an effective inhibitor of inflammation in experimental animal models.

#### Example 9 - Inhibition of osteoclast differentiation by the NBD peptide

The processes of bone morphogenesis and remodeling require the maintenance of a balance between the synthesis of bone matrix by osteoblasts and the resorption of bone by osteoclasts (Suda *et al.*, (1992) Endocr. Rev. 13, 66-80; Suda *et al.*, (1999) Endocr. Rev. 20, 345-357). Bone-resorbing osteoclasts are multinucleated giant cells that differentiate from myeloid precursors and various soluble factors including colony stimulating factor-1 (CSF-1), Interleukin-1 (IL-1), Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6 and IL-11 (Suda *et al.*, (1992) Endocr. Rev. 13, 66-80; Suda *et al.*, (1999) Endocr. Rev. 20, 345-357) that affect osteoclast differentiation at distinct stages. One factor that is critical for osteoclastogenesis is the recently described molecule named RANKL (receptor activator of NF- $\kappa$ B ligand) that is also known as ODF (osteoclast differentiation factor), OPGL (osteoprotegerin ligand) and TRANCE (TNF-related activation-induced cytokine) (Kong *et al.*, (1999) Nature, 397, 315-323; Lacey *et al.*, (1998) Cell 93, 165-176; Suda *et al.*, (1999) Endocr. Rev. 20, 345-357; Wong *et al.*, (1999) J. Leukoc. Biol. 65, 715-724; Yasuda *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95, 3597-3602). The receptor for RANKL is a member of the TNF-receptor family named RANK (receptor activator of NF- $\kappa$ B) (Anderson *et al.*, (1997) Nature 390, 175-179; Dougall *et al.*, (1999) Genes Dev.

13, 2412-2424) and binding of RANKL induces NF- $\kappa$ B activation (Anderson *et al.*, (1997) Nature 390, 175-179; Darnay *et al.*, (1998) J. Biol. Chem. 273, 20551-20555; Darnay *et al.*, (1999) J. Biol. Chem. 274, 7724-31; Suda *et al.*, (1999) Endocr. Rev. 20, 345-357; Wong *et al.*, (1998) J. Biol. Chem. 273, 28355-28359). Moreover, osteoclast  
5 differentiation is dependent upon NF- $\kappa$ B activation and gene-targeting studies have demonstrated that mature osteoclasts fail to develop in mice lacking the p50 and p52 NF- $\kappa$ B subunits (Franzoso *et al.*, (1997) Genes Dev. 11, 3482-3496).

Osteoporosis is a severely debilitating disease characterized by an extensive loss of bone mass that is mediated by osteoclast-dependent bone resorption (Suda *et al.*,  
10 (1992) Endocr. Rev. 13, 66-80; Suda *et al.*, (1999) Endocr. Rev. 20, 345-357). It is therefore possible that selective inhibition of NF- $\kappa$ B activation in osteoclast precursor cells would prevent osteoclast differentiation and provide the basis for therapeutically effective drugs for the treatment of osteoporosis. Therefore the effect of the NBD peptides on osteoclast differentiation was tested using a previously described *in vitro*  
15 model (Jimi *et al.*, (1999) Exp. Cell Res. 247, 84-93). Mouse bone marrow cells plated into 48-well tissue culture trays were incubated with human macrophage-colony stimulating factor (M-CSF; 20 ng/ml) and human RANKL (100 ng/ml) for six days in the absence or presence of various concentrations (6.25, 12.5 and 25 mM) of either mutant or wild-type NBD peptide. The cells were then fixed and stained for the osteoclast  
20 phenotypic marker tartrate-resistant acid phosphatase (TRAP) and TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Triplicate samples were counted and results were calculated as means  $\pm$  SD. As shown in Figure 7 the wild type but not mutant peptide dose-dependently inhibited osteoclast differentiation.

25 This data demonstrates that disruption of the core IKK complex by a cell permeable NBD peptide that inhibits NF- $\kappa$ B activation prevents RANKL-induced osteoclast differentiation indicating that drugs specifically targeting the NBD will be effective for the treatment of osteoporosis. As an extension of these *in vitro* studies, the

same peptides can be analyzed for their effects on osteoporosis *in vivo*. Ovariectomized mice (Charles River Labs) that exhibit severe osteoporosis are treated with the NBD peptides and the effects on bone density over a timecourse of treatment determined.

5 Example 10 - Effect of NBD peptides on other NF-κB mediated disorders

In addition, it is also possible to examine the effects of the NBD peptides on asthma. NF-κB activation in bronchiolar epithelial cells, T-cells and bronchiolar macrophages has been observed in the airways of asthmatic patients and in animal models of asthma (Ray & Cohn, (1999) J. Clin. Invest. 104, 985-993; Christman *et al.*, (2000) Chest 117, 1482-1487). In addition, many agents that induce asthma cause NF-κB activation and many of the genes that encode proteins involved in asthma (*i.e.*, leukocyte adhesion molecules, various chemokines, inducible nitric oxide synthase) are NF-κB-dependent. An established mouse model of asthma (Kleeberger *et al.*, (1990) Am. J. Physiol. 258, 313-320) can be used to test the effects of aerosol administration of the NBD peptides on progression of these conditions associated with asthma.

In a similar manner, the effects of the NBD peptides on septic shock can also be measured. Septic shock involves the expression of many NF-κB dependent genes (*i.e.*, TNF, IL-1) that are induced by bacterial endotoxins such as lipopolysaccharide (LPS). LPS comprises the major constituents of the cell walls of gram-negative bacteria and is highly immunogenic and stimulates the production of endogenous pyrogens IL-1 and TNF (Sell *et al.*, (1996) Immunology, Immunopathology & Immunity, Appleton & Lange). To test the effects of the NBD peptides on septic shock, mice are injected with the NBD peptides and-LPS and the survival of animals assessed.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety. The results of the experiments

disclosed herein are being submitted to the journal Science, the manuscript of which is herein incorporated by reference in its entirety.

002280" 0922E4960